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Title: Glucocorticoid Exposure During Hippocampal Neurogenesis Primes Future Stress Response by Inducing Changes in DNA Methylation.

Short title (max. Length 50 char.): Glucocorticoids induce DNAm changes during neurogenesis.

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Abstract

Prenatal stress exposure is associated with risk for psychiatric disorders later in life. This may be mediated in part via enhanced exposure to glucocorticoids (GCs), known to impact neurogenesis. We aimed to identify molecular mediators of these effects, focusing on long-lasting epigenetic changes. In a human hippocampal progenitor cell (HPC) line, we assessed the short- and long-term effects of GC exposure during neurogenesis on mRNA expression and DNA methylation (DNAm) profiles. GC exposure induced changes in DNAm at 27,812 CpGs and in the expression of 3,857 transcripts (FDR≤0.1 and FC expression ≥|1.15|). HPC expression and GC-affected DNAm profiles were enriched for changes observed during human fetal brain development. Differentially methylated sites (DMSs) with GC exposure clustered into four trajectories over HPC-differentiation, with transient as well as long-lasting DNAm changes. Lasting DMSs mapped to distinct functional pathways and were selectively enriched for poised and bivalent enhancer marks. Lasting DMSs had little correlation with lasting expression changes, but were associated with a significantly enhanced transcriptional response to a second acute GC-challenge. A significant subset of lasting DMSs was also responsive to an acute GC-challenge in peripheral blood. These tissue-overlapping DMSs were used to compute a poly-epigenetic score that predicted exposure to conditions associated with altered prenatal GCs in newborn's cord blood DNA. Overall, our data suggest that early exposure to GCs can change the set-point of future transcriptional responses to stress by inducing lasting DNAm changes. Such altered set-points may relate to differential vulnerability to stress exposure later in life.

Significance Statement: Prenatal stress exposure is associated with a wide range of health problems later in life. This may be mediated in part via glucocorticoid (GC) exposure during fetal development known to impact neurogenesis and induce epigenetic changes. Using a human fetal hippocampal progenitor cell line to assess the effects of GCs, we observe that exposure to GCs early during neurogenesis results in lasting changes in methylation (DNAm). Lasting DNAm alterations are associated with a significantly enhanced transcriptional response to a subsequent GC exposure. Our data suggest that early exposure to GCs changes the set point of future transcriptional responses to stress by inducing lasting DNAm changes. Such altered set points may relate to differential vulnerability to stress exposure later in life.

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Introduction

Early life is one of the most important and sensitive periods during the development of an individual (1). Exposure to stress during this critical period, as early as prenatally, has been associated with a wide range of health problems later in life such as increased reactivity to stress, cognitive deficits, psychiatric and behavioral problems (1). In addition to alterations in fetal growth and neurobehavioral development (2), several studies have linked exposure to prenatal stress to structural and connectivity changes in the offspring brain (3, 4). One of the possible mechanisms mediating the negative effects of prenatal stress could be increased fetal exposure to glucocorticoids (GCs) (5–7). Over the course of normal gestation, there is a physiological rise of 2- to 4-fold in maternal GCs that is important for proper fetal growth and maturation. GC exposure of the fetus is tightly controlled by a number of mechanisms, including the metabolism of GCs in the placenta by the 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (8). Maternal prenatal stress, depression and anxiety have been associated with biological changes that could increase fetal exposure to GCs above the required physiological levels. While a number of studies have reported increased plasma cortisol in women experiencing stress, depression or anxiety during pregnancy, this effect is far from consistent (9). Maternal stress has been proposed to be associated with increased GC exposure of the fetus via reduced placental metabolism of cortisol to inactive metabolites by 11β-HSD2 (8). In addition, prenatal stress has also been linked to changes in the offspring's hypothalamic-pituitary-adrenal (HPA) axis with increased and prolonged HPA-axis reactivity consistently observed in animal studies with similar effects, although less pronounced, described in humans (6, 9).

While likely not the sole mechanism explaining the adverse outcomes following exposure to prenatal stress, excessive exposure to GCs above the physiological level may contribute to the observed neurodevelopmental consequences. Although GCs are essential for fetal brain maturation, the developing brain has been shown to be especially vulnerable to excessive GCs, with lasting effects on cognition and cortical thickness reported (9). Effects of GC on neuronal progenitor cells have been identified as potential mediators of these effects (5, 10). Hippocampal neurogenesis, in particular, is of importance as this brain region plays an essential role in regulating the negative feedback loop of the HPA-axis. In mice, a single dose of dexamethasone (DEX), a synthetic GC, at embryonic day 15.5 decreased hippocampal volume and cell proliferation in the subgranular zone of the dentate gyrus in pups and impaired long-term depression and hippocampal neurogenesis in adult mice (11). In macaques, prenatal DEX exposure as well as prenatal stress reduced hippocampal volume and neurogenesis (12, 13). This is supported by *in vitro* data, where reduced neuronal proliferation and differentiation was observed in human multipotent hippocampal progenitor cells (HPCs) after DEX treatment as well as high doses of cortisol (14).

The molecular mechanisms of how prenatal GC exposure might induce these long-lasting changes on neurogenesis and brain structure are largely unknown. There is accumulating evidence, however, that

epigenetic mechanisms are likely to play a major role in mediating these effects (15). At the molecular level, GCs bind to glucocorticoid (GR) and mineralocorticoid receptors (MR), which function as transcription factors (TFs) and regulate gene expression in multiple tissues (16). In addition to altering gene transcription, GR activation can induce changes in DNA methylation (DNAm) (17, 18). Local de-methylation at GC responsive elements (GREs) has been reported following GR stimulation, possibly mediated by activating base excision repair mechanisms (19). This reduction in DNAm likely changes accessibility of the DNA to transcriptional regulators and impacts future transcriptional responses (20).

Exposure to prenatal stress or GCs has been associated with persisting changes in DNAm in neuronal tissues and cells. In animal models of prenatal stress, lasting changes in DNAm in the hypothalamus or hippocampus have been reported in specific candidate genes (21, 22). Another set of studies have reported the impact of chronic administration of GCs on DNAm in adult mouse hippocampus as well as in a rodent primary neuronal cell line, both in candidate genes (17, 23) and at a genome-wide level (18, 24). Here, we extend these previous studies and systematically investigate the impact of GCs on genome-wide DNAm and gene expression in human HPCs undergoing neuronal differentiation (14). We examine how GC exposure at different stages, including proliferation, differentiation and post-differentiation, affects DNAm, and whether these changes are persistent. A special focus is placed on developmental DNAm and gene expression trajectories and how these mechanisms are altered by GC exposure during different developmental periods as well as the interconnection of DNAm and gene expression changes across time. Finally, we map the observed epigenetic changes in HPCs to measures in developing human tissues and assess their potential as biomarkers for prenatal GC exposure.

Results

Effects of DEX treatment during neurogenesis.

To assess the immediate and long-lasting effects of GR activation on gene expression and DNAm during neurogenesis, DEX treatment was applied at four different experimental time points in HPCs (**Fig. 1A** and **SI Appendix, SI Methods**) followed by mRNA and DNA hybridization onto Illumina arrays. Cells were first treated with DEX (1 μM) or vehicle (ETOH) only during the proliferation phase (Pro, 3 days) or during both the proliferation and neuronal differentiation phases (Pro-diff, 10 days). To assess long-lasting effects of DEX, cells treated during proliferation and differentiation stages were cultured for an additional 20 days without DEX (Pro-diff + washout (WO)). To compare DEX effects pre- and post- differentiation, cells were also treated with DEX or vehicle post-differentiation for 10 days followed by 20 days of WO (Post+WO). Clustering the HPCs gene expression profiles from the vehicle treatments with hippocampal gene expression data from embryonic to adult post-mortem brains of the Human Brain Transcriptome atlas (25) we observed that these cells most resemble second trimester pregnancy hippocampal gene expression (**SI Appendix, SI Results** and **Fig. S1**).

Using immunohistochemistry, we previously reported that DEX treatment (1 μM) in HPCs decreases proliferation and differentiation of progenitor cells (14). Here, we predicted the proportion of neuronal, glial and doublecortin-positive (DCX) cells across treatments using the CellCODE algorithm (26). As previously described (14), DEX significantly decreased neuronal and DCX-positive cell proportions during the proliferation and/or differentiation phases as compared to the vehicle condition. However, the decrease in neuron, glial and DCX+ cell proportions did not persist after washout, indicating that these immediate effects are reversed within 20 days of further culture (**SI Appendix, SI Results, Fig. S2A** and **S2B**).

DEX-induced changes in gene expression and DNAm during neurogenesis

We identified significant gene expression changes in 3,512 unique transcripts (FDR≤0.1 and absolute FC≥1.15; **SI Appendix, Table S1**) following DEX treatment across the four different time points. The majority of the changes were observed during proliferation (Pro, n=2389 transcripts or 68%) and differentiation (Pro-diff, n=1409 or 40%) (**Fig. 1B**). Only a small number of differentially expressed probes (DEPs) showed long-lasting DEX effects following washout, both in pre- and post-differentiation treatments (Pro-diff+WO, n=348 or 6% and Post+WO, n=212 or 0.2%, respectively) indicating that for the majority of the transcripts, changes were not maintained after the removal of DEX. Even though a much smaller number of DEPs was identified following washout (Pro-diff+WO), significant overlaps were observed with DEPs from the earlier time points (Pro vs. Pro-diff+WO n=80 and fisher exact p-value=7.79×10^{-5}; Pro-diff vs. Pro-diff+WO, n=70 and fisher exact p-value=6.17×10^{-11}), but not with the post-differentiation time point (**SI Appendix, Fig. S3A**). The same pattern was observed for analyses on the probe- as well as at the gene level (**Fig. 1B** and **SI Appendix, Fig. S3B**).

Significant DEX-induced DNAm changes were identified in 27,812 unique CpGs (FDR≤0.1; **SI Appendix, Table S2**) across all time points. As for gene expression, the majority of differentially methylated sites (DMSs) were identified in cells treated in the proliferation stage (Pro, 65.5% of total DMSs) and minimal effects of DEX were seen when cells were treated post-differentiation (Post+WO, 1.1% of total DMSs, **Fig. 1C**). In contrast to the effects on gene expression, a significantly larger proportion of CpG sites (24.4% of the total DMSs) showed long-lasting DNAm changes after washout (p-value<2.2×10^{-16} based on test for equality of proportions). This was not the case when the cells were treated after differentiation, here a significantly lower proportion of DMSs (1.1%) was observed (Post+WO, proportion test p-value<2.2×10^{-16}). DMSs identified following washout (Pro-diff+WO) shared a significant overlap with DMSs identified at the earlier time points, especially when mapped to genes (at the gene level: Pro-diff vs. Pro-diff+WO n=874 and fisher exact p-value<2.2×10^{-16}, and Pro vs. Pro-diff+WO n=3,194 and fisher exact p-value<2.2×10^{-16}; **SI Appendix, Fig. S3C** and **S3D**).

DEX-induced DMSs have distinct trajectories during neurogenesis.

To follow up on our observation that changes in DNAm seem to be coordinated to some degree across developmental stages, we sought to determine whether these changes cluster in different DNAm trajectories across neurogenesis. We applied the Gene Activity in Patterns Sets (GAPS) algorithm (27) to identify the main trajectories by clustering the DNAm profiles of the top DMSs (FDR≤0.1 and absolute DNAm change ≥5%, n=792; **SI Appendix, Table S3**). We identified four trajectories across our experimental conditions where 566 CpG sites were found to be uniquely associated with a specific trajectory (**Fig. 2A**, left panel). Across differentiation, DNAm levels at these DEX-responsive sites either decrease (green trajectory, n=71 CpGs), increase (red trajectory, n=127 CpGs) or remain relatively stable (blue and beige trajectories, n=330 and 38 CpGs, respectively). The effects of DEX on DNAm, while significant for each single CpG for at least one time point, often showed differences in the direction (more or less methylated). In the blue and the beige trajectories, but not the two other trajectories, significant DEX-induced differences in average DNAm levels were observed (**SI Appendix, Fig. S4)**. For 24% of the sites across all 4 trajectories, DEX-induced significant methylation changes that occur early in neurogenesis are maintained following the washout of DEX. At this time point (Pro-diff+WO), CpGs in the beige trajectory show the largest effects. We next mapped these 566 sites to their closest genes and performed enrichment analysis in Gene Ontology (GO) categories. Overall, genes mapped to these DNAm trajectories are involved in cellular and organ development, transcription, neurogenesis and neuronal differentiation (**Fig. 2B**). For the majority of the genes (72%), we observe the expected inverse correlation between DNAm and mRNA expression profiles during proliferation and differentiation. For 142 of the transcripts mapped to the individual DNAm trajectories, DEX induced significant changes in mRNA expression during the proliferation and/or differentiation stages (see **Fig. 2C** for examples) but this was only observed for 18 transcripts following washout (Pro-diff+WO). The lack of concomitant mRNA expression and DNAm changes following the washout of DEX is also evident for all DMSs showing lasting DNAm changes (6,895 CpGs), where only 2.6% of the associated transcripts (4,368 transcripts) show long-lasting expression changes. The top DMS showing the largest long-lasting demethylation change (-20.1%, cg14284211) from the beige trajectory is located in the *FK506 binding protein 5* (*FKBP5*) locus. Fine mapping of additional CpGs in this locus using targeted bisulfite sequencing show similar long-lasting demethylation across multiple GREs of this locus (**SI Appendix, SI Results, Table S4** and **Fig. S5**).

To better understand what may drive these changes in DNAm, we tested if gene expression of enzymes involved in DNAm processes are affected by DEX at the different time points. qRT-PCR results show that TET1 and UHRF1, but not other enzymes, are significantly upregulated by DEX in the Pro-diff treatment stage after correcting for relative changes in neuron, glial and DCX+ cell proportions (**SI Appendix, Fig. S6)**.

Functional annotation of DEX-induced DMSs.

To annotate the biological functions of the DEX-induced changes observed, we performed multi-level ontology analysis (MONA) combining the results from DNAm and mRNA expression to identify common Gene Ontology biological processes between the two datasets. This analysis revealed an enrichment in pathways involved in neurogenesis as well as in the regulation of transcription across our time points (**SI Appendix, SI Results** and **Fig. S7A**). Interestingly, a set of pathways was exclusively associated with DNAm changes occurring in the Pro-diff+WO condition with associated gene expression changes at this time point as well as in the earlier time points. These were axon development, actin filament organization, negative regulation of cell proliferation, small GTPase mediated signal transduction, and neuropeptide signaling pathways. This indicates that biological functions associated with lasting DNAm changes show earlier differential mRNA expression after DEX during proliferation and differentiation.

We next aimed to characterize the regulatory function of the genomic locations of our DEX responsive DMSs. Using GR ChIP-Seq peaks from ENCODE lymphoblastoid cell lines exposed to DEX, we observed significant enrichment within GREs for DMSs in Pro-diff and Pro-diff+WO (p-values<0.001, OR=1.59 and 1.25, respectively) while Pro and Post+WO treatments were not enriched for GR binding sites (**Fig. 3A**). Using the 15-states ChromHMM annotation of the Roadmap Epigenomics project for hippocampal tissue (28), we observed that DMSs of Pro, Pro-diff and Pro-diff+WO treatments are enriched within enhancers and flanking active transcription start site (**Fig. 3A)**. Interestingly, an overrepresentation of multiple bivalent and/or poised states characterized by the presence of both activating and repressive histone marks was exclusively observed for the long-lasting DMSs (**Fig. 3A** and **SI Appendix, Fig. S7B**).

DNAm changes induced by prolonged GC exposure during neurogenesis are associated with enhanced responsivity of target transcripts to a subsequent acute GC challenge.

The above presented analyses showed that early DEX exposure only leads to minimal lasting gene expression changes, but to substantial changes in DNAm within regulatory regions. Such changes in DNAm may poise the target transcripts to a more exaggerated transcriptional response to a subsequent activation of the GR. To test this hypothesis, we used a combination of treatments with the early 10 days exposure to 1 μM DEX, followed by the 20 days of washout and a single acute challenge of DEX at a lower concentration (100 nM) for 4 hours and compared it to a single acute challenge of DEX in cells treated with vehicle during the early 10 days exposure (see schema **Fig. 3B** and **SI Appendix, SI Results**). We focused this analysis on all transcripts that mapped to a CpG showing long-lasting DNAm changes (n=3,852 transcripts nearby 6,895 Pro-diff +WO CpGs). We identified 702 transcripts (18.2%) with significant changes in gene expression after the additional acute challenge of DEX in comparison to cells treated with vehicle (Pro-diff+WO+acute, FDR≤0.1 and FC≥|1.15|; **SI Appendix, Table S5**). This

fraction was substantially higher than the one previously observed in Pro-diff+WO without the acute stimulation (n=86 transcripts or 2.4%), or in cells exposed to the same acute challenge but treated with vehicle during proliferation and differentiation (n=254 transcripts or 7.1%; **SI Appendix, Table S6**). In addition, these transcripts exhibited an overall larger magnitude of change in gene expression following the second acute challenge (mean absolute FC=|1.29| ± 0.19, range from -3.25 to 2.86) as compared to minimal non-significant changes observed in the Pro-diff+WO treatment without acute challenge (mean FC=|1.06| ± 0.07, range from -1.42 to 1.72, p-value Wilcoxon test<$2.2x10^{-16}$) or the acute challenge alone (mean FC=|1.22| ± 0.1, range from -1.93 to 1.74, p-value Wilcoxon test<$2.2x10^{-16}$, **Fig. 3B and SI Appendix Fig. S8**). Together these results indicate that at least a subset of the long-lasting DMSs prime neighboring loci to be more responsive to subsequent GR activation.

Interestingly, the lasting DMSs associated genes with an increased response to subsequent DEX exposure (702 transcripts mapping to 1,282 CpGs) showed stronger enrichment among previously reported DMSs regulated during fetal development (29) as compared to all the long-lasting DMSs (Pro-diff+WO+acute permutation p-value=0.004 OR=1.3 compared to Pro-diff+WO permutation p-value<0.001, OR=1.23; **SI Appendix, SI Results**).

Cross-tissues relevance of DEX induced differential DNAm and potential as biomarker.

Although GR-responsive changes in DNAm are likely to be largely tissue-specific (20), overlapping DNAm changes have been reported in specific loci and may serve as biomarkers of exposure in peripheral tissues as observed in mice (23). To test this, we performed an enrichment analysis between the lasting DMSs in HPCs and DEX associated DNAm changes in human blood cells from the MPIP cohort (n=113). In this dataset, we identified 26,264 CpGs with significant changes in DNAm (FDR≤0.01 and absolute change in DNAm ≥2%) after correcting for confounders including cell type proportions. We observed a significant overlap of 496 sites between these DEX-responsive CpGs (permutation p-value<0.001 and OR=1.1976; **Fig. 3C** and **SI Appendix, SI Results, Fig. S9** and **Table S7**).

We next wanted to test whether the lasting DNAm changes in HPCs with common DEX-induced changes in peripheral blood could serve as biomarker for prenatal GC exposure in newborns. For this purpose, we used data from 817 newborns and their mothers within the Preeclampsia and Intrauterine Growth Restriction (PREDO) longitudinal cohort (30). We focused our analyses on pregnancy conditions related to higher prenatal GC levels: prenatal treatment with betamethasone, a synthetic GC, as well as the cumulative severity of maternal depression and anxiety symptoms throughout pregnancy. Using the overlapping 496 GC-responsive CpGs in blood and HPCs, we computed a weighted poly-epigenetic score

using an elastic-net regression which selected 24 CpG sites within 24 distinct loci. The weights were determined from the DEX-associated changes in peripheral blood (MPIP cohort) with the majority displaying reduced methylation after DEX (**SI Appendix, SI Results, Fig. S9** and **Table S8**). Lower weights were associated with higher de-methylation in blood following DEX exposure (β=0.077, p=0.04; **SI Appendix, Fig. S10**). Applying this combined GC-responsive poly-epigenetic score to DNAm measured in cord blood, we observed a significant association of this score with maternal anxiety (β=-0.0011, SE=0.00054, p=0.044, **Fig. 3D**) and maternal depression (β=-0.0015, SE=0.00066, p=0.022, **Fig. 3E**), with a lower poly-epigenetic score observed in newborns exposed to higher depressive or anxiety symptoms. No significant association was seen with betamethasone treatment (β=-0.0039, SE=0.019, p=0.84), but here, only a small number of newborns (n=35) were exposed to pre-delivery betamethasone treatment. However, as expected, the direction of the associations of betamethasone exposure, maternal depression and anxiety with the score was the same.

Discussion

Using a human fetal HPC line, we observed that exposure to GCs during proliferation and differentiation, but not once the cells are differentiated, results in lasting changes in DNAm (**Fig. 1C**). These lasting DNAm changes are not correlated with strong baseline changes in gene transcription, but with an enhanced responsivity of the target transcripts to a second GC challenge (**Fig. 3B**). This suggests that early exposure to GCs may have a lasting impact on nervous system development not only by altering proliferation and neuronal differentiation rates as previously reported (5), but also by priming relevant transcripts to an altered transcriptional response upon subsequent GR activation. The induction of such poised or metaplastic states could then contribute to the increased risk for behavioral problems and psychiatric disorders observed with prenatal GC exposure (9). In fact, the level of DNAm of these lasting DMSs is regulated during human fetal brain development especially for those linked to altered gene expression to a subsequent GC exposure. Moreover, when we used a subset of the DMSs showing lasting effects in HPCs and acute effects in blood to compute a GC-responsive poly-epigenetic score in newborns' cord blood DNA, this score showed significant associations with maternal depression and anxiety (**Fig. 3D and 3E**). This could suggest that the findings of our *in vitro* model may translate to human pregnancy and that DMSs with cross-tissue effects could serve as biomarkers for conditions associated with prenatal GC exposure.

Unique functional role of lasting DNAm changes.

The lasting DMSs identified were distinct from the other DMSs with only a limited overlap on the CpG level with DMSs following treatment during proliferation and differentiation (n=180 overlapping CpGs or 2.6%; **SI Appendix, Fig. S3C**). Indeed, unique GO terms relevant for the function of differentiated neurons were identified for genes mapped to these DMSs (**SI Appendix, Fig. S7A**). This suggests that within the lasting DMSs, there could be at least two major categories, one related to differences in

neurodevelopment and the other to functional differences in mature cells. Prenatal GCs could thus not only impact on neuronal proliferation and differentiation as such, but also change the sensitivity of more mature cells or tissues to stress exposure later in life. Indeed, an altered sensitivity to postnatal stressors following prenatal stress exposure termed metaplasticity has been proposed as model for how prenatal environments may impact long-term risk trajectories (9, 31). This model suggests that different adaptive physiological responses to stress in individuals could be poised by prenatal stress (here we suggest via epigenetic mechanisms) but triggered by various postnatal environments giving rise to the observed variety of short- and long-term phenotypic outcomes (see SI Appendix, SI Discussion for a detailed description of this adaptive model).

In line with this model, the lasting DMSs were also enriched for a specific subset of chromatin marks (see **Fig. 3A**) including bivalent/poised TSS, flanking bivalent and bivalent enhancers. Bivalent/poised chromatin states are characterized by the presence of both activating and repressive chromatin marks and are associated with paused RNA polymerase II (RNA PolII) that can be quickly released into productive transcription, a common feature of stress-responsive genes in yeast but also observed in humans (32). Previous work investigating chromatin accessibility induced by GR activation identified a subset for which heightened sensitivity was retained as a “memory” of the hormone induction following withdrawal (33). In line with these observations, our results of enhanced gene expression changes following a subsequent GCs exposure for a subset of these long-lasting DMSs would suggest that these sites allow the cell to adjust its transcriptional response dependent on previous exposure. Although bivalency has been observed in differentiated tissues, it is important to note that the Roadmap data used for the enrichment analysis originate from bulk hippocampal tissue and that our DNAm profiles in HPCs are also from a cell mixture. Therefore, we cannot differentiate whether these sites are indeed localized at bivalent/poised state of the same nucleosome or in different cells harboring one or the other chromatin marks. Nevertheless, the fact that the long-lasting DMSs are enriched among these regulatory marks and associate with altered expression following a subsequent exposure to GCs suggest a role for these sites in regulating or priming future gene expression responses to GCs, be it in a cell type specific manner or within the context of a mixed tissue, with distinct GC-sensitivities. These effects could thus alter the set-point of ensembles of cells to future stress exposure.

Molecular mechanisms inducing DNAm changes.

What could be the mechanisms driving these lasting DNAm changes? GC-induced changes in DNAm may be direct downstream effects of GC-action at the respective enhancer elements, but may in part also be secondary to altered proliferation and differentiation observed following DEX treatment. In our previous work using a GR antagonist (14) as well as an inhibitor of *SGK1* (34), an activator of GR, we have shown that both treatments block DEX-induced reduction in proliferation, providing evidence that at least some of these changes maybe more directly downstream of GR activation. From our data we observed that lasting

DNAm were enriched in GREs and for these sites only, we observed a larger fraction of de-methylation vs. hyper-methylation after DEX (**SI Appendix, Fig. S7B**). This is concordant with prior studies that have described local DNA de-methylation at GREs with GR activation (18), likely mediated by induction of base excision repair mechanisms (19). However, DNA de-methylation was not the rule for the lasting DNAm changes across all sites, with enhancers, bivalent/poised sites and TSS flanking sites showing similar proportions of hyper- as well as de-methylation (**SI Appendix**, **Fig. S7B**), similar to previous observations (18, 24). The fact that changes of DNAm were observed in both directions is also in line with our data showing that mRNA levels of both enzymes associated with de-methylation (TET1) as well as re-methylation (UHRF1) were affected by DEX following treatment during proliferation and differentiation (**SI Appendix, Fig. S6**). In contrast, changes in mRNA expression of TET1 and UHRF1 as well as differences in cell type proportions were not observed anymore following the 20 days of washout although differences in DNAm are observed. These results suggest that long-lasting DNAm changes are not the result of strong and sustained global expression changes in epigenetic writers. However, they might result from a locus-specific recruitment and/or activation of these enzymes in response to GCs initiated in a small number of cells/alleles during proliferation and differentiation and continue to spread after the removal of DEX. Indeed, although not significant, we observed the same direction of changes in DNAm at the earlier time points (Pro and/or Pro-diff) for 54% of the long-lasting DMSs.

Cross-tissues GC-responsive CpGs as biomarker for prenatal exposure.

Lastly, we wanted to understand whether lasting changes in DNAm in our *in vitro* model would also be observed in human blood. While previous studies in mice have shown that GC-induced DNAm changes are mostly tissue specific (18), overlapping changes have been reported and may be aggregated in those GR-responsive enhancers with common functionality across tissues (23). We also identified a subset of lasting HPC DMSs that were also acutely responsive to DEX in peripheral blood (n=496 CpGs, **Fig. 3C**). In addition to be predictive of maternal stress exposures when combined into a GC-responsive poly-epigenetic score, these cross-tissues CpGs were also significantly enriched in DNAm changes observed in cord blood of newborns exposed to pre-delivery administration of the synthetic GC, betamethasone, as well as maternal anxiety and depression (**SI Appendix, SI Results** and **Table S7**). A number of studies have reported that the two latter conditions might also be accompanied by increased fetal GC exposure, by either increasing maternal GC, decreasing placental GC metabolism or activating the offspring's HPA-axis (5, 6). Although not directly tested in our newborn cohort, maternal prenatal stress may also impact the newborn's DNAm profiles via other systems, such as immune activation with reciprocal interactions of the immune and the stress systems (35, 36).

These DNAm changes in newborn may be markers for risk, as betamethasone exposure has been shown to be associated with mental health problems in children (44) and conditions associated with altered fetal GC exposure, including maternal depression and anxiety but also infections have been associated with a

number of neurodevelopmental abnormalities (6, 9, 35). The fact that our cross-tissues GC-responsive poly-epigenetic score significantly predicted both the severity of maternal prenatal depression as well as anxiety suggests that these sites could serve as biomarkers for prenatal GC exposure (**Fig. 3D** and **3E**). Lower scores reflecting more de-methylation following GC exposure (**SI Appendix, Fig. S10B**) were associated with exposure to higher maternal depressive and anxiety symptoms over pregnancy. The direction of association, together with the overlapping findings from prenatal betamethasone treatment, would be in line with higher GC exposure in offspring of mothers with prenatal anxiety and depression. Given that prenatal GC levels were not measured in the PREDO cohort, we cannot directly test this proposition. It is also important to note that although we observed significant associations, the small effect sizes (β=-0.0011 for maternal anxiety and β=-0.0015 for maternal depression) are indicative that only a very small portion of the variance in symptoms is explained by the cross-tissues GC-responsive poly-epigenetic score (R^2=0.6% for maternal anxiety and 0.3% for maternal depression) and would likely have small, clinically not relevant predictive power. Additional work is needed to further develop the score as well as replicate these associations in additional longitudinal cohorts with measure of GCs as well as early intervention studies to assess its ability to predict change in postnatal stress exposure.

Conclusions

Overall, our data suggest that GC-induced DNAm reflects a complex pattern of changes likely related to effects on proliferation and differentiation as well as lasting changes in more mature tissues. These lasting changes may specifically target pathways important for neuronal transmission and prime target genes to an altered responsivity to subsequent GC exposure. By this, prenatal exposure to GCs could not only alter neurodevelopmental trajectories but also change the set-point of stress-reactivity of adult tissues. Together these two factors could influence and increase the risk for psychiatric disorders.

Materials and Methods

Materials, experimental procedures and data analysis for the culture and gene expression and methylation profiling of immortalized, multipotent human fetal HPC line HPC03A/07 as well as methylation profiling in blood samples of the Max Planck Institute of Psychiatry (MPIP, n=113) and PREDO (n=817) cohorts are described in SI Appendix, SI Materials and Methods. The MPIP cohort study protocol was approved by the local ethics committee and all individuals gave written informed consent. The PREDO study protocol was approved by the Ethical Committees of the Helsinki and Uusimaa Hospital District and by the participating hospitals. A written informed consent was obtained from all women. Data from the HPC gene expression microarray experiments were deposited at the GEO repository, GSE119842 and GSE119843 and HPC methylation data at GSE119846.

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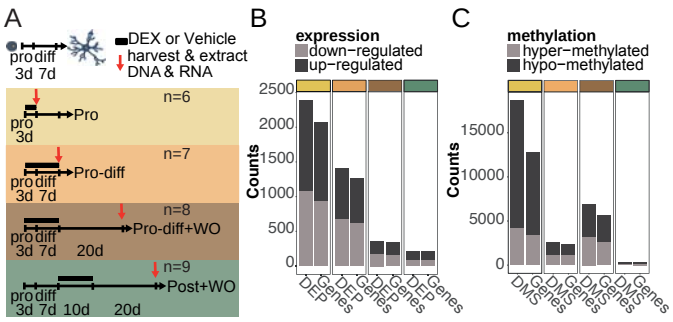
Figure legends

Figure 1. DEX-induced changes in gene expression and DNA methylation across treatments. (A) Schema illustrating the different treatments with Vehicle or DEX (1 μM) applied to HPCs across neurogenesis. (B) Number of differentially expressed probes (DEP) and (C) differentially methylated sites (DMS) induced by DEX across treatments. The bar on the left represent the number of significant probes from the array and on the right the number of genes mapped to these probes for each treatment.

Figure 2. Top DEX-induced differentially methylated sites cluster into four distinct trajectories during neurogenesis. (A) DNAm of the vehicle (dashed line) and DEX treatments across our experimental conditions for DMSs belonging to each trajectory identified by GAPS algorithm. The average DNAm and SEM overall sites within each trajectory appear in bold. (B) Top significantly enriched clusters of GO biological process terms for genes mapped to DMSs within each trajectory. (C) Boxplot of the methylation levels of a representative CpG site for each trajectory and its associated gene expression levels across treatments.

Figure 3. Functionality of the long-lasting differentially methylated sites induced by glucocorticoid receptor activation. (A) Heatmap of enrichment results for GR-Chip-seq binding sites and predicted ChromHMM states for each treatment (colors display fold enrichment and stars indicate significant permutation p-values <0.05). (B) Violin plot showing the fold change (DEX - vehicles) in gene expression for each treatment condition of the 3,852 closest transcripts that map to CpGs showing long-lasting DNAm changes (Pro-diff+WO, n=6,895 CpGs). Below a schema illustrating the previous Pro-diff+WO treatment and the two acute challenge treatments applied. Significant transcripts for each treatment condition are marked in red. (C) Overlap of DEX-responsive DMSs in HPCs (Pro-diff+WO) and human peripheral blood cells of the MPIP cohort. Associations between maternal (D) depression (β=-0.0015, SE=0.00066, p=0.022) and (E) anxiety (β=-0.0011, SE=0.00054, p=0.044) during pregnancy and the poly-epigenetic score computed for 817 newborns' cord blood DNA samples.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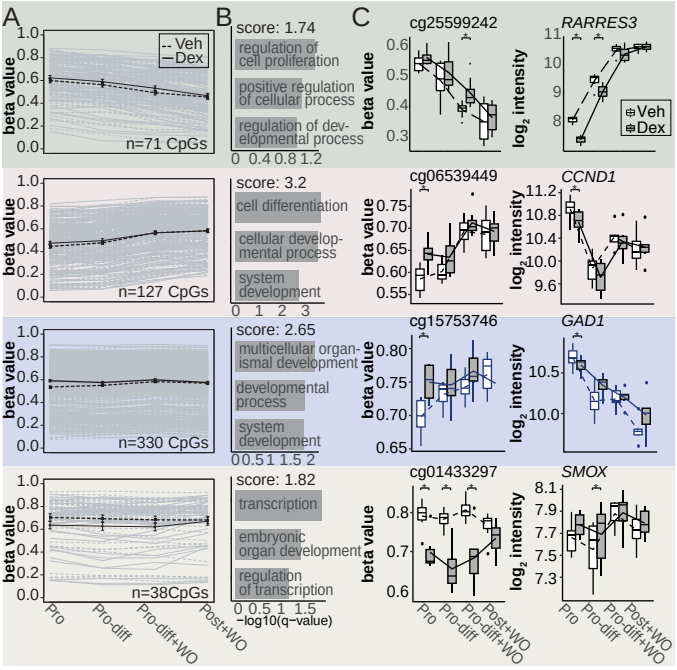
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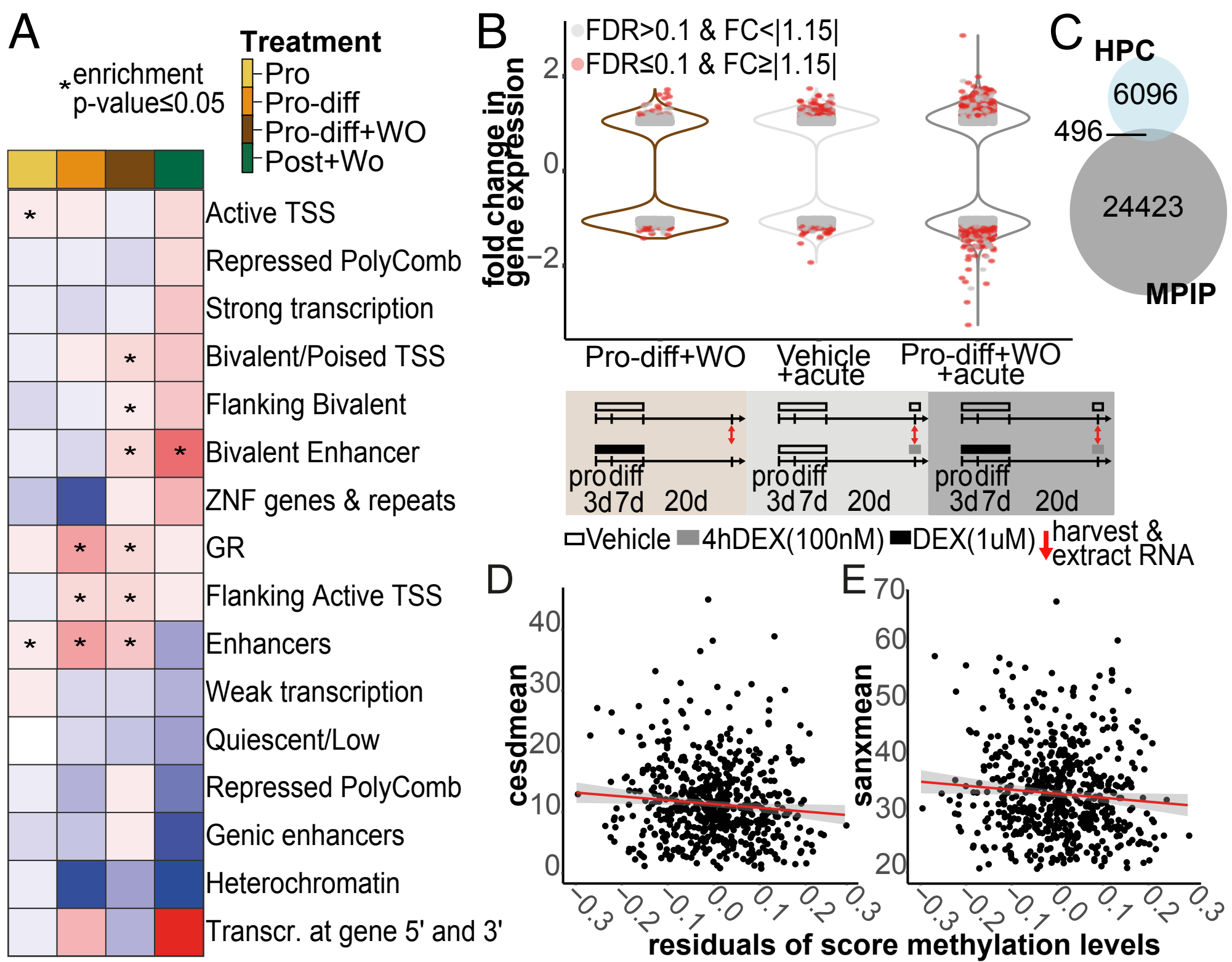
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Active TSS
Repressed PolyComb
Strong transcription
Bivalent/Poised TSS
Flanking Bivalent
Bivalent Enhancer
ZNF genes & repeats
GR
Flanking Active TSS
Enhancers
Weak transcription
Quiescent/Low
Repressed PolyComb
Genic enhancers
Heterochromatin
Transcr. at gene 5' and 3'
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FDR≤0.1 & FC≥|1.15|
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DEX(1uM)
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